It is likely that the developmental reduction of the female gametophyte, from a significant nourishing tissue for the embryo in nonflowering seed plants to the structurally reduced embryo sac of angiosperms, was compensated for by the origin of endosperm as a nutritive tissue for the embryo (character advances 4 to 6, Fig. 5). If double fertilization in *Ephedra* and angiosperms was inherited from a common ancestor, the evolution of triploid endosperm, and not double fertilization per se, would appear to have been the significant reproductive innovation, in part, responsible for the evolutionary and ecological radiation of angiosperms.

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24. This is consistent with recent speculation on the topic by M. J. Donoghue [*Evolution* 43, 1137 (1989)], who expands on similar suggestions by earlier workers.

25. I thank R. Wyatt, B. Palevitz, and J. Symmes for critical reading of the manuscript, T. Reagin for histological preparations and graphics, and M. Camann for assistance with photography. Supported by NSF Research Grant BSR 8818035.

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Novel Fluorogenic Substrates for Assaying Retroviral Proteases by Resonance Energy Transfer

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The 11-kD protease (PR) encoded by the human immunodeficiency virus 1 (HIV-1) is essential for the correct processing of viral polyproteins and the maturation of infectious virus, and is therefore a target for the design of selective acquired immunodeficiency syndrome (AIDS) therapeutics. To facilitate the identification of novel inhibitors of HIV-1 PR, as well as to permit detailed studies on the enzymology and inhibition of this enzyme, a continuous assay for its activity was developed that was based on intramolecular fluorescence resonance energy transfer (RET). The assay used the quenched fluorogenic substrate 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL)–Ser Gln Asn Tyr Pro Ile Val Gln–5-[(2-aminoethyl)amino]naphthalene-1 sulfonic acid (EDANS), whose peptide sequence is derived from a natural processing site for HIV-1 PR. Incubation of recombinant HIV-1 PR with the fluorogenic substrate resulted in specific cleavage at the Tyr-Pro bond and a time-dependent increase in fluorescence intensity that was linearly related to the extent of substrate hydrolysis. An internally quenched fluorogenic substrate was also designed that was selectively cleaved by the related PR from avian myeloblastosis virus (AMV). The fluorescence quantum yields of the HIV-1 PR and AMV PR substrates in the RET assay increased by 40.0- and 34.4-fold, respectively, per mole of substrate cleaved. Because of its simplicity, rapidity, and precision in the determination of reaction rates required for kinetic analysis, this method offers many advantages over the commonly used high-performance liquid chromatography– or electrophoresis-based assays for peptide substrate hydrolysis by retroviral PRs.

As with other classes of positive-strand RNA viruses, retroviruses encode proteins that are initially synthesized as large polyprotein precursors and are later processed by posttranslational cleavage (*1*). Translation of the polycistronic viral mRNA results in the synthesis of two precursor polyproteins: Pr^{gag}, which contains the structural capsid proteins, and $Pr^{gag\text{-}pol}$, which contains information for both the structural proteins and the replicative enzymes. The *env* gene products are translated as a precursor polyprotein from a separately spliced mRNA transcript. Retroviruses also encode a small, 10- to 12-kD protease (PR) that is generally expressed as part of the $Pr^{gag\text{-}pol}$ precursor, except in the case of the avian retroviruses, where it is synthesized as the COOH-terminal portion of Pr^{gag} (*2*). The retroviral PR is required for the processing of both the Pr^{gag} and $Pr^{gag\text{-}pol}$ precursor polyproteins at specific cleavage sites. These cleavages are believed to occur during or just after virion assembly and have been shown for HIV-1 and murine leukemia virus (MuLV) to be required for the maturation of infectious virus particles (*3*). Thus, inhibition of the viral PR has become an important target for the design of antiretroviral agents, including new therapeutic agents for AIDS.

Three-dimensional crystal structures of the PRs for Rous sarcoma virus (RSV) (*4*) and HIV-1 (*5*) have verified predictions (*6, 7*) that the retroviral PRs are structurally and functionally related to the eukaryotic aspartic proteinase family of enzymes. Strategies used to design inhibitors of human renin, a related aspartic proteinase that specifically cleaves angiotensinogen to angiotensin I and initiates a hypertensive response, are now being applied to the design of HIV-1 PR inhibitors. A variety of techniques have been previously used to measure retroviral proteolytic activity, including protein immunoblot analysis of the *gag* polyprotein and its cleavage products (*7, 8*), and high-performance liquid chromatography (HPLC) (*9, 10*) or thin-layer electrophoretic (*11*) analysis of synthetic peptide-cleavage fragments. All of these methods are relatively time-consuming and impractical for screening and characterizing large numbers of inhibitors. In addition, they are not well suited for enzymological studies because continuous measurement of reaction kinetics is not possible (*12*).

As an alternative approach, we have developed a fluorescence assay based on the quenched fluorogenic substrates **1** and **2** (Fig. 1A). These substrates consist of an

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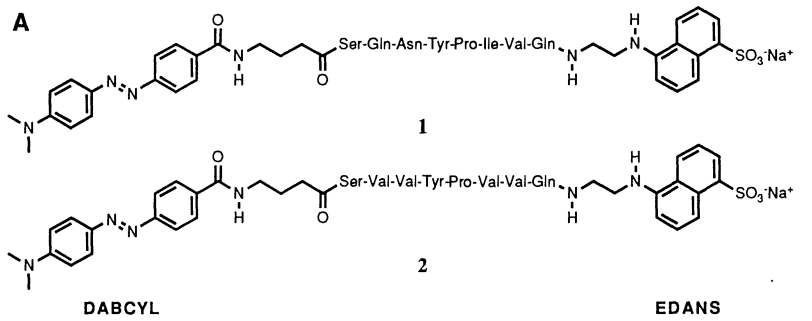

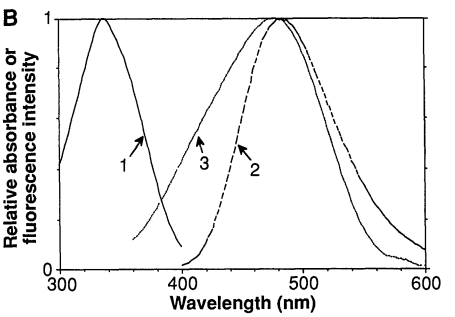

Fig. 1. (**A**) Structures of fluorogenic substrates **1** (for HIV-1 PR) and **2** (for AMV PR). The fluorescent donor is EDANS and the quenching acceptor is DABCYL linked via a GABA spacer. Octapeptides (Multiple Peptide Systems) were derivatized with conventional condensation chemistry of commercially available DABCYL and EDANS with the octapeptide NH_2 and COOH-termini, respectively (*32*). Purification was accomplished by reversed-phase HPLC with a linear water-to-acetonitrile gradient at *p*H 4.9; the GABA-Ser linkage is mildly unstable below *p*H 3. Verification of the final products was obtained by amino acid analysis, nuclear magnetic resonance, and mass spectrometry. (**B**) The absorption (line 1) and technical fluorescence (line 2) spectra of free EDANS, and the absorption spectrum of free DABCYL-GABA (line 3). The peak molar extinction coefficients in the assay buffer (Fig. 2, legend) are $5438M^{-1}$ cm^{-1} at 336 nm (EDANS) and $15{,}100M^{-1}$ cm^{-1} at 472 nm (DABCYL).

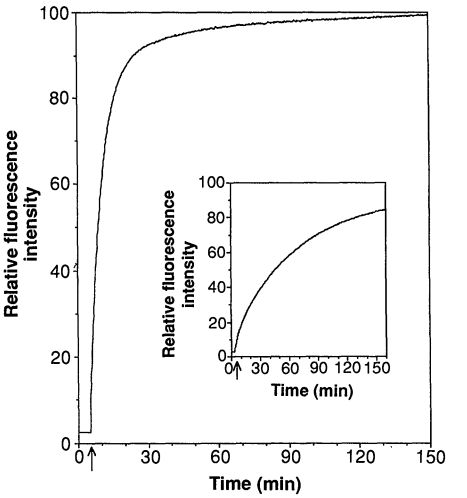

Fig. 2. Hydrolysis of fluorogenic substrate **1** by HIV-1 PR at 37°C, as monitored by steady-state fluorescence (*33*). The reaction was carried out with 10.7 μ*M* substrate **1** at *p*H 4.7 in a buffer containing 0.1*M* sodium acetate, 1*M* NaCl, 1 m*M* EDTA, 1 m*M* dithiothreitol, 10% dimethyl sulfoxide, and bovine serum albumin (1 mg/ml), in a volume of 120 μl (*34*). The arrow denotes the addition of recombinant HIV-1 PR to a final concentration of 35 n*M* (*35*). (**Inset**) Analogous reaction carried out with 5 μ*M* substrate **2** and AMV PR in the medium described for HIV-1 PR, except buffered at *p*H 6.0 by 0.1*M* sodium phosphate. At the arrow, AMV PR was added to a final concentration of 33 μg/ml.

octapeptide with a fluorescent donor, 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid (EDANS), and a quenching acceptor, 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL), attached at the COOH- and NH_2-termini, respectively. The octapeptide sequence of substrate **1**, SQNYPIVQ (*13*), corresponds to the naturally occurring $Pr55^{gag}$ p17/p24 cleavage site for HIV-1 PR, which cleaves at the Tyr-Pro peptide bond. This sequence was selected because it had been shown to be a better substrate than synthetic peptides based on other cleavage sites (*9, 10*). The sequence used for substrate **2**, SVVYPVVQ, although not a natural cleavage sequence for avian myeloblastosis virus (AMV) PR, contains a high Val content, which was predicted to enhance substrate efficiency based on an examination of the various cleavage sites on the AMV polyprotein (*14*). A γ-aminobutyric acid (GABA) spacer was inserted between the DABCYL group and the NH_2-terminal Ser in both substrates to avoid potential steric hindrance of substrate binding by the bulky acceptor. The intrinsic fluorescence of EDANS is dramatically reduced in these substrates because of intramolecular resonance energy transfer (RET) (*15*) to the DABCYL group. Since RET becomes insignificant beyond distances of about 100 Å, the full fluorescence quantum yield of EDANS is restored after cleavage of the peptide and concomitant liberation of the DABCYL-linked peptide fragment. Hence, proteolytic activity can be continuously monitored by simply recording the increase in fluorescence intensity with time. Furthermore, this increase is linearly related to the rate of hydrolysis of the fluorogenic substrate because for each mole of substrate that is cleaved, the total fluorescence is incremented by the net intensity caused by 1 mol of newly created EDANS-linked peptide fragment.

The fluorescence-monitored hydrolysis of substrate **1** by HIV-1 PR is presented in Fig. 2. The reaction was allowed to proceed to virtual completion, with a fluorescence enhancement of 40.0-fold per mole of substrate cleaved (*16*). (When fluorogenic peptides with nonspecific sequences were incubated with HIV-1 PR, no change in fluorescence intensity was observed.) The cleavage of substrate **2** by AMV PR resulted in a fluorescence enhancement of 34.4-fold (Fig. 2, inset). At substrate concentrations of 3.4 μ*M*, AMV PR cleaved substrate **2** at least 50 times faster than substrate **1**, whereas HIV-1 PR cleaved substrate **1** about ten times faster than substrate **2** (*17*).

HPLC analysis of the products formed in the HIV-1 PR reaction of Fig. 2 indicated complete disappearance of the 53.5-min peak (substrate **1**) and the concomitant appearance of two new species with retention times of 36.4 min and 47.2 min (Fig. 3, A and B). These results suggest that the fluorogenic substrate had been cleaved quantitatively at a single bond (*18*). The absorption spectrum for each peak was also acquired on-line during the chromatography (*19*). The 36.4-min peak exhibited an absorption spectrum of EDANS alone and a fluorescence-yield characteristic of the unquenched EDANS fluorophore, whereas the 47.2-min species was nonfluorescent and exhibited an absorption spectrum representative solely of the DABCYL group. These cleavage products were further subjected to amino acid analysis. The fragment eluting at 36.4 min was identified as PIVQ-EDANS, and the 47.2-min fragment as DABCYL-SQNY. Confirmation of the product identities was obtained by chemically synthesizing the predicted products and by finding that they exhibited retention times on reversed-phase HPLC that were indistinguishable from the HIV-1 PR–liberated fragments (*17, 20*). These analyses proved unequivocally that proteolysis of substrate **1** occurred specifically at the Tyr-Pro bond.

A typical velocity-versus-substrate concentration profile for the hydrolysis of substrate **1** by HIV-1 PR is presented in Fig. 4. Under our standard high ionic strength assay conditions at 30°C (*21*), classical Michaelis-Menten kinetics were observed, with $K_m \pm SD = 103 \pm 8$ μ*M* and $V_{max} \pm SD = 164 \pm 7$ n*M* min^{-1}; assuming the catalytic unit to be a dimer, these results gave a catalytic rate (k_{cat}) of 4.9 s^{-1}. In previous reports where synthetic peptide substrates for HIV-1 PR were studied, K_m values in

the millimolar range were generally obtained (*10, 22, 23*). An exception is the dodecapeptide YVSQNYPIVQNR, with a reported K_m of 60 μM (*24*). Darke *et al.* (*21*) reported a K_m of 1.5 mM for the octapeptide SQNYPIVQ at *p*H 5.5 and at low ionic strength. It would thus appear that the presence of the DABCYL and EDANS groups confers improved substrate-binding properties on the octapeptide in this assay. Consistent with this conclusion is the observation that hydrolysis of fluorogenic substrate **1** was suppressed only 15% in the presence of equimolar unlabeled peptide SQNYPIVQ (*20*).

Pepstatin is a potent inhibitor of many cellular aspartic proteinases and is a reported inhibitor of HIV-1 PR (*8, 22, 24, 25*). A Dixon plot yielded an inhibition constant (K_i) for pepstatin of 17 nM (Fig. 5). When the data was plotted according to the method of Cornish-Bowden (Fig. 5, inset), parallel lines were obtained, showing that inhibition by pepstatin is purely competitive in nature. Pepstatin inhibited the cleavage of substrate **2** by AMV PR fairly weakly in the fluorescence assay, with a median inhibitory concentration (IC_{50}) of about 40 μM at *p*H 6 (*17*). Values for K_i or IC_{50}, which were previously reported for pepstatin inhibition of HIV-1 PR, ranged from about 0.4 to 250 μM (*8, 22, 24, 25*). A number of factors are probably responsible for the apparent disparity in these values, including differing substrates, assay conditions, and preparations of HIV-1 PR. For routine measurements of HIV-1 PR activity, a fluorogenic substrate concentration of 2 to 5 μM provides adequate signal to noise on any laboratory-grade fluorometer. This concentration is well below the K_m of 103 μM. Thus, a novel consequence of the high sensitivity of the RET assay with regard to HIV-1 PR inhibitor screening is that the IC_{50} values provided by this assay are good approximations for K_i when the inhibition is competitive (*26*).

The feasibility of applying RET to the measurement of hydrolase activity was shown by Latt *et al.* and Carmel *et al.* (*27*) nearly two decades ago. However, the use of this concept (or analogous quenching and cleavage strategies) for the design of efficient PR substrates has limited practical application (*28*). Selection of an efficient donor/acceptor (D/A) energy transfer pair is critical for obtaining a large change in fluorescence signal per mole of cleaved substrate. The latter characteristic enables rate determinations from the hydrolysis of a very small fraction of total substrate, which is highly desirable for estimating initial reaction velocities. Previous applications used substrates in which the fluorophore and quencher were separated by no more than four amino acid residues. For significantly greater separation distances, relatively poor quenching would be expected for the types of fluorescent and quenching groups (especially those based mainly on collisional or short-range quenching mechanisms) that were typically used in these studies. We began with the premise that a minimum peptide length of about seven amino acids would be necessary for efficient substrate recognition and cleavage by HIV-1 PR (*10*). The choice of EDANS and DABCYL as an optimal D/A pair was made on the basis of a number of considerations. (i) There is ex-

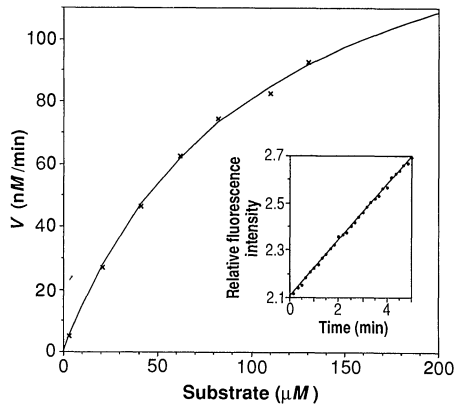

Fig. 4. Hydrolytic reaction rates at 30°C as a function of substrate **1** concentration. Fluorescence measurements (*33*) were carried out in the *p*H 4.7 assay buffer with 1.12 nM HIV-1 PR. Rates were derived from the initial 5 to 8 min of the reaction, always corresponding to less than 1% hydrolysis of the total substrate. Each data point represents the average of three velocity (V) determinations; the SD of the measured rates in these and in replicate measurements performed in other experiments was always better than 2%. The nonlinear least-squares fit to the Michaelis-Menten equation is shown by the solid line. Although the mediocre solubility of **1** (*36*) limited the measurements to a maximum substrate concentration of only 1.3 times the K_m, the excellent fit and the reproducibility of the data lend confidence to this analysis. (**Inset**) Initial phase of the hydrolysis used for rate determinations. The data shown correspond to the 20 μM substrate reaction. The digitized points represent signal averaging over 10-s intervals, and the least-squares linear fit to the data is indicated by the solid line. Conversion of the reaction velocities from fluorescence intensity per unit time (V_i) to concentration (molarity) per unit time (V_c) was performed by the following procedure, which simultaneously corrected for the inner filter effect (*37*). At each fluorogenic substrate concentration used for a rate measurement, a known low concentration of free EDANS (C_{ref}) was added (in the absence of HIV-1 PR), and its apparent fluorescence intensity was determined (I_{ref}) under instrumental conditions identical to those used in the rate measurement. The converted and corrected velocity was then calculated from $V_c = V_i \times (C_{ref}/I_{ref}) \times (e/(e - 1)) \times R_{ref}$, where e is the factor by which the substrate quantum yield is enhanced after cleavage, and R_{ref} is the ratio of the intensities of equimolar solutions of reference compound to that of the fragment PIVQ-EDANS (*38*).

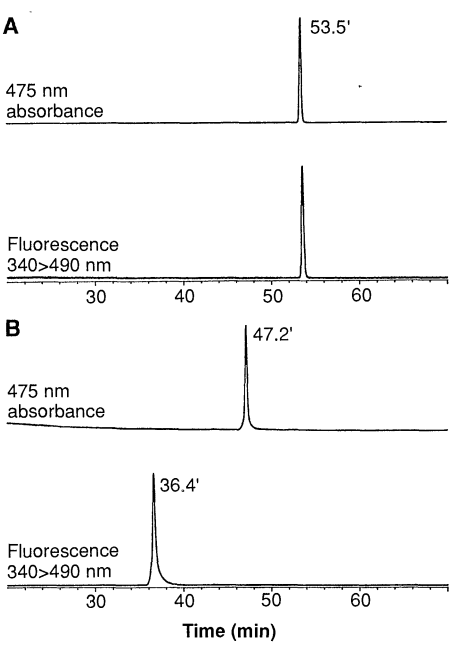

Fig. 3. HPLC analysis of fluorogenic substrate and reaction products. Overlayed are the fluorescence and 475-nm absorbance chromatograms resulting from a linear elution gradient of 5 to 55% acetonitrile, 1% per minute (*19*). (A) Fluorogenic substrate **1**, before addition of HIV-1 PR. (B) Products of substrate 1 after hydrolysis by HIV-1 PR for 2.5 hours under the conditions described in Fig. 2. The eluates that produced the peaks were collected for amino acid analysis.

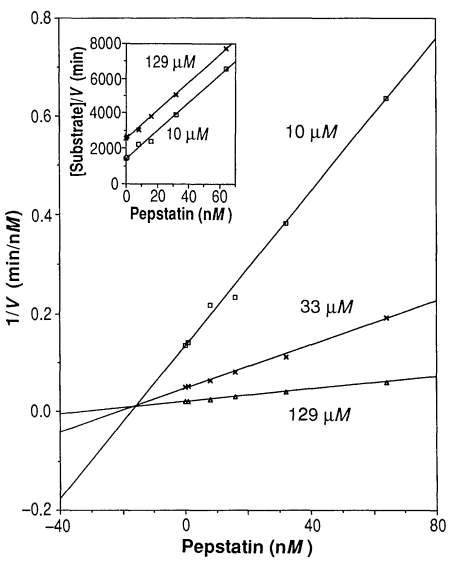

Fig. 5. Inhibition of HIV-1 PR by pepstatin at *p*H 4.7, 30°C. The reaction conditions were identical to those used in Fig. 4. Pepstatin (Calbiochem) was dissolved in DMSO, and diluted into the assay buffer just before rate measurements were made. The data were taken at three concentrations of substrate **1** (indicated on plot) and are shown in the form of a Dixon plot; the point of intersection yields K_i of 17 nM. (**Inset**) Cornish-Bowden plot (*39*).

tremely good spectral overlap of EDANS emission with the strong visible absorption band of DABCYL (Fig. 1B), leading to very efficient energy transfer. R_0, the Foerster distance for 50% energy transfer efficiency, was estimated to be 33 Å for this D/A pair (*29*). (ii) The use of a relatively long-lived donor, such as EDANS, leads to improved suppression of residual substrate fluorescence, since RET is significantly enhanced by diffusion of donor and acceptor moieties. In substrates **1** and **2**, the maximal D/A separation distance (R) is expected to be about 29 Å (assuming an extended octapeptide configuration). Hence, R is always less than R_0, and the efficiency of energy transfer increases steeply with decreasing R for distances $R < R_0$. [For example, 20-fold quenching of donor fluorescence would be achieved at $R = 0.61\ R_0$, and 65-fold quenching at $R = 0.5\ R_0$ (*30*).] (iii) The detectability of EDANS is enhanced by several factors, including its moderately good quantum yield, reasonable stability against photobleaching, and a Stoke shift of over 100 nm (Fig. 1B). (iv) The water solubility of EDANS is beneficial for solubilizing hydrophobic peptides such as those that are good substrates for HIV-1 PR. (v) The well-separated visible absorption band of DABCYL facilitates substrate purification and quantitation as well as the analysis of the substrate fragments produced by proteolysis. The fact that DABCYL is also nonfluorescent improves the detectability of EDANS fluorescence, since it allows the latter to be measured with cutoff filters or very wide band-pass monochromator slits or filters (*31*).

In summary, we have shown that fluorescence RET can be effectively applied to assaying retroviral PRs even when relatively large peptides are used as substrates. The simplicity, rapidity, and precision of the RET assay facilitates its adaption to a variety of uses and formats. For large-scale screening, the RET assay lends itself readily to automated formats, such as the 96-well fluorescence plate reader. The RET method may also be used to study substrate specificity requirements for retroviral proteases, since an efficient D/A pair can be used with virtually any peptide sequence, requiring only that the donor and acceptor groups can be positioned so as to minimally perturb the enzyme-substrate interaction. Examination of modeled substrate-enzyme complexes, on the basis of known three-dimensional crystal structures of several viral and eukaryotic aspartic proteinases, indicates that the bulky D/A groups on the fluorogenic substrate should not interfere with the binding of the peptide moiety to its complementary enzyme subsites. Thus, in principle it should be feasible to design fluorogenic peptide substrates specific for cleavage by other retroviral proteases, as well as by the structurally related eukaryotic aspartic proteinases such as renin, chymosin, and the pepsins.

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12. After this manuscript was submitted, a report by N. T. Nashed *et al.* [*Biochem. Biophys. Res. Comm.* **163**, 1079 (1989)] appeared describing a continuous spectrophotometric assay for HIV-1 PR based on a substrate with the chromogenic Phe(NO_2) residue at the cleavage site.
13. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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16. The molar fluorescence enhancement was also estimated by HPLC analysis of samples of the HIV-1 PR–substrate **1** reaction mixture at times before the hydrolysis was complete. Integration of the peaks of the fluorescence and 475 nm absorbance chromatograms (Fig. 3) yielded an apparent 33-fold enhancement of the fluorescence quantum yield of PIVQ-EDANS over the parent substrate **1**. However, the value of 40.0 obtained under assay conditions is likely to be more accurate, since the HPLC chromatography, although performed at a similar *p*H (4.9), was carried out in an acetonitrile-water solvent system. The latter medium alters the photophysical properties of the probes and the conformation of the peptide, which in turn affects the degree of quenching caused by RET.
17. E. D. Matayoshi, unpublished observations.
18. Confirmation that complete hydrolysis was achieved in the HIV-1 PR reaction (Fig. 2) was also obtained directly from fluorescence lifetime measurements of the fluorogenic substrate, before and after incubation with HIV-1 PR. RET quenching of the excited state of EDANS is eliminated upon cleavage of substrate, resulting in a considerable lengthening of the fluorescence lifetime. Fluorescence lifetimes were measured by the methods of time-correlated single-photon counting (TCSPC) (PRA System 3000; Photochemical Research Associates) and phase fluorometry (SLM Model 4800 three-frequency instrument; SLM-Aminco). Measurements were made with 340-nm excitation, and the broadband emission was defined by a Schott KV418 cutoff filter. A scattering solution or short lifetime reference sample was used for obtaining the excitation pulse profile (TCSPC), or as a phase and modulation reference (phase method). We relied on TCSPC for carrying out detailed multicomponent analyses, whereas the phase method was used primarily for rapid lifetime measurements or as additional confirmation of the single-photon counting results. The fluorescence decay of the substrate fragment PIVQ-EDANS in the assay buffer was found to be monoexponential with a lifetime of 13.0 ns. In contrast, the decay of the uncleaved substrate **1** was dominated by components with short lifetimes and was highly heterogeneous, reflecting the numerous solution conformations of the octapeptide, which give rise to a distribution of intramolecular distances and energy transfer rates between EDANS and DABCYL. When the decay was analyzed as the sum of three discrete exponential components, an acceptable fit was obtained with lifetimes of 0.68 ns, 2.34 ns, and 9.47 ns and respective exponential amplitudes of 0.531, 0.128, and 0.011 (implying that about 87% of the integrated intensity was due to the two shortest components). However, further analysis suggested that a fit to a distribution of lifetimes would be more appropriate. When comparing these results with the apparent 40-fold quenching observed under steady-state conditions, it should be borne in mind that the most highly quenched EDANS components (shortest lifetime) also have the smallest intensities. In addition, the flash lamp excitation used in these measurements limits the shortest reliably resolved lifetime in a heterogeneous decay to about 0.3 ns. Hence, the apparent decay components that were obtained very likely correspond only to substrate conformations in which the rate of energy transfer is less than maximal (E. D. Matayoshi, in preparation).
19. Analytical HPLC, as well as the isolation of substrate fragments, was performed on a Hewlett-Packard 1090M system equipped with diode array absorbance and fluorescence detectors, using either a Brownlee 250 × 4.6 mm C8 silica column at *p*H values below 5, or a Hamilton 50 × 4.1 mm PRP3 polymer column over a *p*H range of 2 to 8. A water-to-acetonitrile gradient was used; either 0.1% trifluoracetic acid (*p*H ~2), 10 m*M* sodium acetate (*p*H 4.9), or 10 m*M* tris-HCl (*p*H 8) was included to vary the *p*H. The detection system permitted simultaneous acquisition of the chromatograms for fluorescence and several absorbance wavelengths, as well as the absorption spectra for all peaks. The fluorescence (excitation at 340 nm, emission at 490 nm) and 475 nm absorbance signals, which arise from the EDANS and DABCYL groups, respectively, could be used to monitor and quantitate the fragments produced by proteolysis of the substrate.
20. G. T. Wang and G. A. Krafft, unpublished observations.
21. We observed that in the high ionic strength buffer (1*M* NaCl and 0.1*M* NaOAc, *p*H 4.7) the activity of HIV-1 PR (30°C, 3.4 μ*M* substrate **1**) was nearly threefold greater than at low ionic strength (0.1*M* NaOAc, *p*H 4.7). In addition, the cleavage of **1** by HIV-1 PR was maximal at *p*H 4.7. For these reasons, we used the high ionic strength buffer for routine assay conditions. The fragments produced by HIV-1 PR cleavage of substrate **1** at high versus low ionic strength were found by HPLC analysis to be identical (*17*).
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26. For a competitive inhibitor, $(IC_{50}/K_i) = 1 + (S/K_m)$, where S is the substrate concentration.
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29. R_0 was estimated (*15*) with a value of 0.67 for the orientation factor, which is valid when donor and acceptor dipoles undergo a dynamic randomization of relative orientations during the excited state lifetime of the donor. This assumption is reasonable because of the considerable flexibility of the DABCYL and EDANS linkages to the peptide, as well as the flexibility of the peptide itself. A value for the donor quantum yield (Q) of 0.13 was used on the basis of the yield measured for the substrate fragment PIVQ-EDANS, made by comparison to quinine sulfate in 1*N* sulfuric acid ($Q = 0.546$) [J. N. Demas and G. A. Crosby, *J. Phys. Chem.* **75**, 991 (1971)].
30. Assuming a relative intramolecular diffusion coefficient of the ends of substrate **1** to be 5×10^{-7} cm^2 s^{-1} [E. Haas, E. Katchalski-Katzir, I. Z. Steinberg, *Biopolymers* **17**, 11 (1978)], the donor and acceptor could move approximately 13 Å relative to one another during the excited state lifetime of EDANS.
31. In principle, it should be possible to use an acceptor that is also fluorescent and to obtain reaction rates by monitoring the time-dependent decrease in sensitized acceptor fluorescence. In practice, however, obtaining comparable sensitivity by use of this method is more difficult because it requires finding a D/A pair in which (i) the donor can be excited without significant direct excitationof the fluorescent acceptor, and (ii) the acceptor and donor fluorescence spectra are well-separated from one another.
32. G. T. Wang, E. D. Matayoshi, G. A. Krafft, manuscript in preparation.
33. Steady-state fluorescence data were obtained on a Spex DM-1B spectrofluorometer equipped with photon counting and double-grating excitation and emission monochromators. Samples were measured in 3-mm-square microcells at the desired temperature. Intensity data was acquired continuously and recorded directly on an interfaced microcomputer. The rate of fluorescent substrate hydrolysis was computed from a linear regression analysis.
34. Assays were routinely run in the presence of 10% dimethyl sulfoxide (DMSO) to aid the solubilization of HIV-1 inhibitors, most of which are highly insoluble in purely aqueous solutions. Although DMSO at this concentration reduces the activity of HIV-1 PR by about 25%, we consider it a necessary experimental compromise in order to ensure maximal dissolution of the inhibitors in a monomeric state. The addition of bovine serum albumin (BSA) at 1 mg/ml was also found to greatly reduce losses of HIV-1 PR, substrate, and some inhibitors by nonspecific absorption to the walls of cuvettes, test tubes, and pipette tips. The BSA does not appear to interact with the fluorophore, as judged from its lack of effect on the fluorescence lifetime of the substrate fragment PIVQ-EDANS. In the DMSO- and BSA-containing buffer, the rate of hydrolysis of **1** was confirmed to be directly proportional to the concentrations of HIV-1 PR (*17*).
35. Recombinant HIV-1 PR was obtained from an *Escherichia coli* expression system and purified to approximately 70% (J. Rittenhouse and R. L. Simmer, manuscript in preparation). The concentration of HIV-1 PR in our dilute stock solutions was estimated by the method of M. Bradford [*Anal. Biochem.* **72**, 248 (1976)], as well as by fluorometric determination of the Trp concentration (2 mol of Trp per mole of HIV-1 PR) in samples of denatured protease in 6*M* guanidinium HCl, with *N*-acetyl tryptophanamide in the same solvent as a reference. Estimates obtained with the former method (40% higher than the latter) were used in the calculations reported here. AMV PR was purchased from Molecular Genetic Resources (Tampa, FL).
36. Other fluorogenic substrates are being investigated in which additional charged residues are included beyond the P4 or P4-min positions, in order to improve substrate solubility (*17, 20*).
37. The inner filter artifact [J. B. F. Lloyd, in *Standards in Fluorescence Spectrometry*, J. N. Miller, Ed. (Chapman & Hall, London, 1981), p. 27] is especially severe for this application because of the presence of two chromophores. Although it can be reduced by the use of fluorescence microcells, the artifact becomes significant at concentrations of **1** greater than about 10 μ*M* even with 3 × 3 mm cells. No inner filter correction is necessary if only the relative rates are to be compared in a series of measurements where the substrate concentration remains fixed (and where the varying component is optically transparent at the excitation and emission wavelengths used), since the multiplicative correction factor is the same for each measurement. However, if one wishes to convert hydrolysis rates to concentration units or to compare rates observed at different substrate concentrations (where the absorbance exceeds about 0.02 unit at either excitation or emission wavelengths), the inner filter correction is required. The correction is constant throughout the actual hydrolysis measurement, since the extinction coefficients as well as the absorption and fluorescence spectra of the donor and acceptor are not altered by hydrolysis of the substrate.
38. Free EDANS is suggested as a reference for the inner filter correction, since it is commercially available and spectrally similar to the PIVQ-EDANS fragment that is liberated by proteolysis. For the procedure discussed in the legend to Fig. 4, the added EDANS must be of sufficiently low concentrations so as to not create an inner filter artifact.
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40. We thank S. Cepa for carrying out HPLC–mass spectrometry, S. Dorwin for the amino acid analyses, and J. Rittenhouse, R. L. Simmer, and coworkers for providing the recombinant HIV-1 PR used in our studies. We are indebted to J. Huffaker for her skilled technical assistance in many of the experiments. This research was supported in part by NIH AI27720.

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Molecular Characterization of a Functional cDNA Encoding the Rat Substance P Receptor

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Substance P is a member of the tachykinin peptide family and participates in the regulation of diverse biological processes. The polymerase chain reaction and conventional library screening were used to isolate a complementary DNA (cDNA) encoding the rat substance P receptor from brain and submandibular gland. By homology analysis, this receptor belongs to the G protein–coupled receptor superfamily. The receptor cDNA was expressed in a mammalian cell line and the ligand binding properties of the encoded receptor were pharmacologically defined by Scatchard analysis and tachykinin peptide displacement as those of a substance P receptor. The distribution of the messenger RNA for this receptor is highest in urinary bladder, submandibular gland, striatum, and spinal cord, which is consistent with the known distribution of substance P receptor binding sites. Thus, this receptor appears to mediate the primary actions of substance P in various brain regions and peripheral tissues.

SUBSTANCE P (SP) IS A NEUROPEPtide considered to function as a neurotransmitter or modulator in the central and peripheral nervous system. Of all neuropeptides, SP is perhaps the best characterized in terms of distribution, sites of release, and biological actions (*1*). SP is a member of a family of structurally related peptides called the tachykinins (*2*). The mammalian tachykinin peptide family currently includes SP, neurokinin A (NKA), neurokinin B (NKB), neuropeptide K, and neuropeptide γ (*3, 4*). SP is produced from any of three differentially spliced mRNAs, and the mature undecapeptide is stored in secretory vesicles and secreted upon cellular stimulation (*5*). SP has excitatory effects on both peripheral and central neurons. It also elicits a variety of biological responses in nonneuronal tissues, including stimulation of smooth muscle contraction, exocrine and endocrine gland secretion, and plasma extravasation and regulation of immune and inflammatory responses (*1, 6*). The stimulation of SP receptors (SPRs) with ligand is followed by an increase in the hydrolysis of phosphatidylinositol 4,5-bisphosphate; this is a result of the G protein–mediated activation of a phosphoinositide-specific phospholipase C (*3, 7*).

We have used molecular cloning techniques in conjunction with expression analysis to determine the primary structure of the rat SPR. The initial isolation of a cDNA fragment encoding part of the SPR was obtained by the use of the polymerase chain reaction (PCR) technique (*8*). Because receptors of the G protein–coupled superfamily display homology in the putative transmembrane domains (*9*), we localized two such areas suspected to be unique for tachykinin receptors on the basis of the deduced sequence of a bovine substance K receptor (SKR) (*10*) and other members of this receptor superfamily. We designed two degenerate oligonucleotides for use as PCR primers (primer PCR II, 5′ TG$^{T}_{G}$ATGGCIGCITT$^{T}_{C}$AA$^{T}_{C}$GC 3′; and primer PCR VII, 5′ ATIGG$^{A}_{G}$TT$^{A}_{G}$TAGATNGT 3′).

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